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14. ABSTRACT

We are studying the utility of using B.fragilis OmpA as a vehicle on which to put antigenic epitopes of organisms that can be used in bioterror, with the aim of eventually constructing a vaccine vehicle vector. OmpA is the major outer membrane protein of B. fragilis, a gram negative anaerobe that normally resides in the gut. There are four homologs for ompA in the genome. The purpose of this study was to construct a B. fragilis ompA deletant and to begin to characterize the function of OmpA, as well as the specific function(s) of the various loops exposed on the surface. Initial studies indicated difficulty in achieving stable expression, export to periplasm and integration of B. fragilis OmpA into E. coli membrane. Thus we optimized a method to insert the recombinant gene directly into B. fragilis. We inserted two restriction sites into a recombinant ompA gene so that we could later replace the internal portion of the gene with one modified to express specific epitopes on loop 2 and loop 3. We will proceed by expressing and detecting the FLAG-tagged OmpAs directly in B. fragilis WAL 186 ompA deletion strain, as well as characterizing the phenotypes of B. fragilis with modified OmpAs.

15. SUBJECT TERMS

None listed.

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Introduction

The dangers of bioterror bacterial or viral agents have been of concern to the military and political leaders for decades. Methods to prevent infection with these agents and to treat infections that may occur are being studied with intensity. Historically, vaccines have been the most efficient method of handling diseases in large populations. Studies of the immune system and vaccine effectiveness have shown that the ideal way to induce a complete immune response of both the mucosal and systemic systems is to administer vaccines in a manner that mimics the natural route of infections. Over the past 15 years, experimental bacterial vaccine vectors have been produced that elicit immune responses against bacterial, viral, protozoan and metazoan pathogens in laboratory animals. Among the advantages of these vaccines is that they are relatively inexpensive to manufacture, they can be given orally, and they can be treated with antibiotics if desired and they effectively induce both humoral and cellular responses. If the organism used as the vector can potentially colonize in the host, the potential of eliciting the appropriate response is increased.

OmpA proteins are among the most conserved of all outer membrane proteins in bacteria; however, the loops expressed on the outer surface are quite variable. Current understanding indicates that the loops are responsible for a variety of virulence characteristics and that they serve as important antigens as well. *Bacteroides fragilis*, most common anaerobic pathogen, is a major component of the stool flora and colonizes the gastrointestinal tract. OmpA is one of the major outer membrane proteins of this organism. Structure/function analysis of the OmpA protein, which will ultimately identify the exposed loops of the protein will both elucidate the role of OmpA in the pathogenic process of *Bacteroides fragilis* and will allow us to exploit the unique nature of this abundant outer membrane protein in designing potential vaccine vectors. These vaccines (i.e., *Bacteroides fragilis* with OmpA modified to express specific epitopes) could be designed for a wide variety of infections that might be acquired by ingestion of food or liquid (including potential biodefense related organisms), and the secretory IgA antibodies produced in the gastrointestinal tract could prevent the pathogen or toxin from exiting the GI tract to invade the circulatory system or other organs.

BODY:

We have made significant and timely progress in the proposed grant. The progress and accomplishments for each Task during the past year is indicated

♦**Task 1: Construct and characterize an *ompA* deletion mutant**

Task 1a: Construction of the *ompA* deletion mutant. √ Completed in Phase 1.

Task 1b: Characterize the deletant strain.

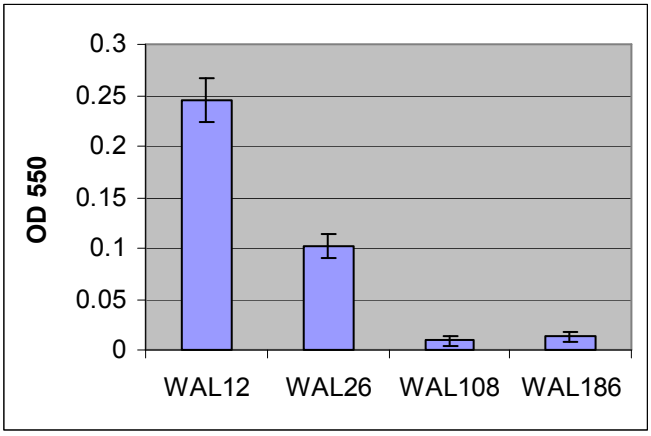
Characterization of the *ompA* deletion mutant. In Phase 1, we demonstrated that the WAL 186 *ompA* deletion mutant was more sensitive to SDS and high salt concentration. Furthermore, both the WAL 108 parental strain and the WAL 186 deletant had small round forms when grown on hyperosmolar media. We also found that while all four *ompA* homologs were transcribed in the parental strain, *ompA4* was not transcribed (nor, obviously *ompA1*) in the deletant strain, indicated that there was negative regulation involved. Strain 186 (*ompA* deletant) is being further characterized by transcriptome analysis (see Task 1d).

Task 1c: Compare parental and *ompA* deletant strains in a rodent abscess virulence model. √ Completed.

Task 1d: Compare parental and *ompA* deletant strains in invasion assays of brain microvascular endothelial cells (BMEC) and macrophages. √ Completed (model adjusted).

Note: We were not able to demonstrate invasion of *B. fragilis* into BMEC. The assay is not performed in an anaerobic chamber, and while *B. fragilis* can survive for lengthy periods in air, it is likely that the *B. fragilis* needs to be metabolically active to be able to invade the BMEC (and metabolism will only occur in anaerobic conditions). Therefore, we set up an alternate assay to look at adherence to intestinal epithelial cells as well as an assay for biofilm formation We found that both the OmpA deletant (WAL 186) and the parental strain (WAL 108) were deficient in 1) adherence to intestinal epithelial cells and

2) biofilm formation compared to strain 638R (WAL 108 is a *thy*⁻ deficient mutant of 638R, engineered to be more amenable to use in allelic exchange experiments).



Absorbance reading of redissolved Crystal violet used to stain the attached cells. WAL 12, B. fragilis type strain ATCC 24285. WAL 26=B. fragilis 638R, parental strain used in genetic manipulations. WAL 108-a thy deficient mutant of B. fragilis 638R; WAL 186: OmpA deletant strain constructed from WAL 108.

We had not expected that a *thy* mutation would have this effect. In order to more closely examine the differences that the *thy* disruption is creating in WAL 108 (since this will be the basic strain for our vaccine vector, we have prepared a cDNA library from RNA from the type strain, 638R (parental strain for WAL 108), WAL 108 (the *thy*⁻ strain used to construct the deletion mutant), WAL 186 (the *ompA* deletant), and WAL 361 (the strain in which the recombinant ompA was inserted back into the chromosome). We submitted these strains for microarray analysis to Nimblegen and expect the results by the end of April 2008. These results will be important in assessing the utility of this strain as the carrier for the vaccine vector.

♦**Task 2: Determine positions of external loops of OmpA**

Task 2a: Tag proposed external loops of OmpA. √ Completed.

Task 2b: Express FLAG-tagged OmpA in *E. coli.*

Task 2c: Express FLAG-tagged OmpA in *B. fragilis*.

Current status: We successfully cloned the OmpA gene into the pet27b vector as indicated in the Report of January 2007. We then needed to express this gene so that it could be properly exported to the outer membrane. We repeatedly tried various permutations in

order to express the *ompA* gene in *E. coli*, but found that the protein expression was toxic to the cells. Although we were able to obtain constructs in which the *ompA* gene was expressed, these clones were unstable. Thus, these experiments have "morphed" into Task 4, and we are using allelic exchange to insert the modified *ompA* genes directly into the chromosome of the *ompA* deletant of *B. fragilis*. Thus, the modified gene will be in exactly the position as the original gene, and will be under the same regulatory controls as the original gene.

♦Task 3: Construct genetically modified OmpAs with specific insertions or deletions in outside loops

Task 3a. Construct OmpAs with specific deletions in outside loops.

Current status: The purpose of Task 3a was to identify those portions of the loops that were responsible for the virulence in the rat abscess model (Task 1C). Since there was no difference in virulence between the parental strain (WAL 108) and the *ompA* deletant (186), there was no need to proceed with Task 3a. (This possibility was discussed in the original proposal).

Task 3b. Construct OmpAs with specific epitopes inserted into outside loops.

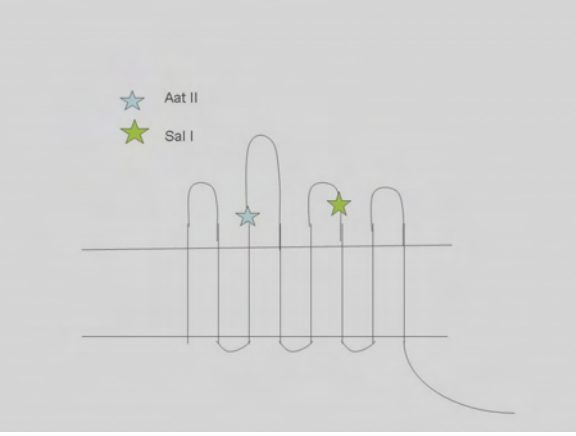
√ Partially completed.

Current status: We decided that it would be very useful to have restriction sites "bracketing" the loop regions of the genes. There were no restriction sites unique to the gene (and not present on the plasmid) in the appropriate positions. Therefore, we planned to insert two restriction sites (AatII and SalI) at the appropriate sites on the *ompA* gene—within loop 2 and one within loop 3. This will allow us to insert the desired epitopes either in Loop 2 or Loop 3 and determine which is the more advantageous. However, the suicide vector, pADB242a, did have one AatII restriction site. Thus, we had to remove this site from the pADB242a vector so that we could then use the vector for further manipulation with the two restriction enzymes. We accomplished this using the NEB Quick blunting kit, creating pADB242amod.

Construction of OmpA-AatII ($OmpA_{R99DVG100}$). We PCR amplified the gene from the beginning of the upstream site (~800 bp upstream of the *ompA* gene) until the beginning of Loop 2 (R_{99}), adding an AatII site to the reverse primer. At the same time,

the downstream portion of the insert (beginning at G_{100} and ending ~800 bp after the end of the *ompA* gene) was PCR amplified, with an Aat II site added to the forward primer. The PCR products were respectively cloned into the TOPO TA cloning vector (Invitrogen) and *E. coli* Top 10 was transformed (separately) with the products. Potential clones were verified with PCR and restriction analysis. The two TOPO constructs (TOPO:: (HindIII)*ompA*up-*ompA*$_{R99}$(AatII) and TOPO::(AatII)*ompA*$_{G100}$—*ompA*down(BamH1) were digested, respectively, with HindIII/AatII and BamH1/AatII. The *ompA* inserts were gel purified and used in a three part ligation with a) pBR322 and b) pADB242amod that had been digested with BamH1 and HindIII. Potential ligated plasmids were used to electroporate electrocompetenet *E. coli* XL Gold supercompetent cells. We obtained positive transformants only with the pBR322 plasmid (we had originally included this plasmid since it is smaller and transforms more easily than pADB242amod.) Positive transformants were identified by restriction analysis and submitted for sequencing. After confirmation of the correct insert, it will be cut out of pBR322 and inserted into similarly digested pADB242Amod, and used for the two part recombination into *B. fragilis* in Task 4b.

Future modification of the vector *ompA* insert. The next step will be to construct modified *ompA* genes with both restriction sites (Aat II in Loop 2 (the longest loop and that most likely to carry the desired epitope) and SalI in loop 3.) Using this modified construct, we will be able to prepare plasmid containing this modified OmpA and digest with AatII and Sal I. The linearized plasmid will then be used in a ligation with a PCR product (relatively small) of an internal piece of OmpA (from the Aat II to the Sal I site). The epitope desired will simply be added right after the AatII site. Potentially, a second epitope could be added on Loop 3 by inserting the sequence right before the Sal I site. The theoretical modified OmpA is depicted below.



Task 3c. Compare parental and modified OmpA in a cytokine induction model. The parental OmpA has been evaluated in a cytokine induction model and a manuscript has been submitted to FEMS Microbiology Letters (the original manuscript was returned with some revisions requested and has been resubmitted.) The submitted manuscript is attached to this report in the Appendix.

Task 3d: Express modified OmpAs generated in Tasks 3a and 3b in *E. coli* and in *B. fragilis*. Current status: Our original assumption was that it would be easier to do all of the modifications in *E. coli*, and then transfer the modified *ompA* gene to a shuttle vector (*pLyl*) to be expressed in *B. fragilis*. Since the OmpA was toxic to *E. coli*, we decided that it would be more efficient and that the results would be more meaningful doing the allelic exchange experiments (described below in Task 4b) with the modified OmpAs.

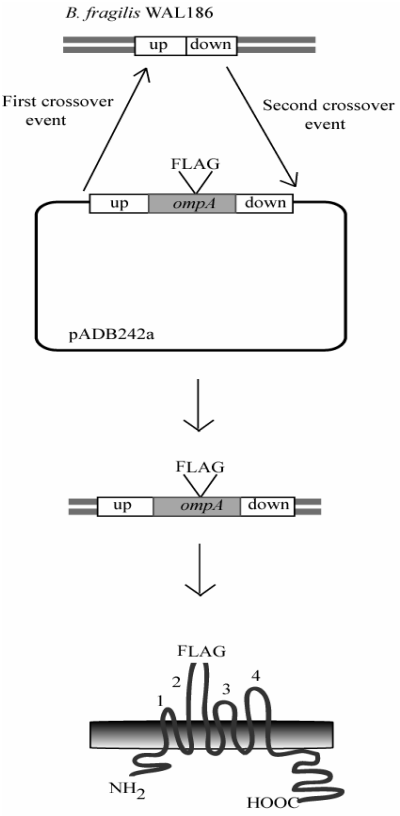
♦Task 4: Construct *B. fragilis* strains with modified OmpAs by allelic exchange using a double crossover technique.

Task 4a. Construct plasmid to be used in allele exchange. **√ Partially completed** When verified, the pBR322 with the assembled ompA insert (with flanking up and down sequences) constructed in Task 3b will be digested with BamH1 and HindIII, and the insert gel purified and religated into digested pADB242a-mod-Aatminus.

Task 4b. Construct *B. fragilis* with modified OmpA. **√ Partially completed**

We have modified our plan for constructing these strains. We are now using our *ompA* deletant strain, constructed during the first phase of these studies, as the parental strain. Using the same two step recombination procedure that was used to construct that strain, we will replace the missing gene with a modified *ompA* gene. This technique has several advantages over gene replacement: After the two step recombination, the only strains with an OmpA protein will have the modified OmpA protein, since we are beginning with a strain lacking this protein. This two step recombination procedure involves a plasmid containing the modified gene, flanked by approximately 800 bp

upstream and downstream sequences, consecutively. After the cointegration and second step recombination, we will be left with either parental strain (ie. ompA deletant) or strains carrying the modified *ompA*.



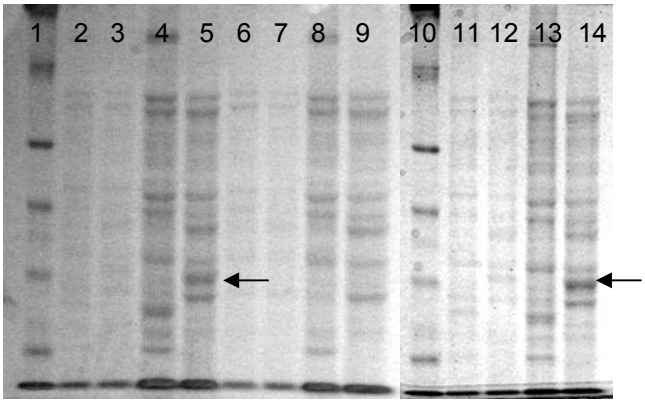
To confirm that this plan will work properly, we first reinserted the recombinant, unmodified ompA gene into the ompA deletant strain (below.)

Cloning and reinserting *ompA1* into *ompA* deletant. The full-length *B. fragilis ompA1* (including about 800 base pairs upstream and downstream of the gene) was cloned in the suicide vector pADB242a. Primers corresponding to the cloned region were used to verify the recombinant plasmid by DNA sequencing. *E.coli* DH5α cells containing pADB242a-up*ompA1*down and E. coli DH5α pK2317 mobilizer plasmids were mated with *B. fragilis* WAL 186 *ompA* deletant. The resulting cointegrants were selected on BHI plates containing gentamycin, rifampicin and tetracycline, and the site of the first recombination was confirmed by PCR. Cointegrants were plated on minimal media with thymine and trimethoprim to select for the second recombination event. PCR amplification of the full-

length ompA1 gene in the resulting trimethoprim-resistant, tetracycline-sensitive strain indicated that ompA1 was restored in this strain.

DNA sequencing and confirmed no sequence differences between the parent strain and the deletant strain in which the *ompA* gene was reinserted. We analyzed the outer membrane protein profile of this strain by SDS-PAGE analysis and confirmed that the OmpA protein is indeed translated.

Crude outer-membranes of the wild type, ompA1 deletant and ompA1 restored strains were analyzed by SDS-PAGE (below). This confirmed that the heat-modifiable OmpA1 was present in the restored strain, demonstrating that our method for genetically manipulating *B. fragilis* to delete and reinsert the *ompA1* gene did not affect its translation. This method will be adapted to introduce recombinant *ompAs* into *B. fragilis* to study their immunogenic properties.



Lane 1: Molecular weight (MW) markers; 2 and 3: *B. fragilis* WAL 108 wild type cell lysates; 4 and 5: WAL 108 wild type Triton pellet (25'C and boiled, respectively); 6 and 7: WAL 186 *ompA* deletant cell lysates; 8 and 9: WAL 186 *ompA* deletant Triton pellet (25'C and boiled, respectively); 10: MW; 11 and 12: WAL 360 *ompA* -restored strain cell lysates; and 13 and 14: WAL 360 *ompA* restored strain Triton pellet (25'C and boiled, respectively).

A two-step double cross-over technique will be then used to perform a gene exchange into *B. fragilis* WAL 186 *ompA* deletant (Baughn and Malamy, 2002). To confirm that the modified loops are exposed in the allele-exchanged *B. fragilis*, the FLAG

epitope displayed in OmpA will be detected by Whole-cell ELISA using monoclonal M2 antibody for FLAG and Proteoqwest Kit (Sigma).

Optimizing electroporation for transforming *B. fragilis*. We are optimizing electroporation as an efficient method to introduce the suicide vector into *B. fragilis*. Electroporation has not been used at all for *Bacteroides*, and thus we are limited to the three part mating technique, which we have used successfully for many allelic exchanges, including the replacement of the ompA gene in the deletion mutant. We are intensively investigating methods of electroporation used for a wide variety of microorganisms. If we are successful at optimizing the electroporation protocol, the time for producing the appropriate strains of *B. fragilis* with modified *ompA* genes for biodefense vaccines can be reduced from a matter of weeks to a matter of days. A successful protocol will not only be very useful for us, but for all of the investigators doing gene manipulation in *B. fragilis*. We hope that we will be able to optimize this procedure within the next few months. However, if we are not successful, we will still be able to complete all of the experiments using the three part mating protocol as we are originally proposed.

Task 4c. **Confirm that modified loops are exposed in allele-exchanged *B. fragilis*.**

Current status: These experiments will commence as soon as Task 4b has been completed. We anticipate that Task 4b will be completed with four months, and once begun, task 4c should be completed within three to six months (i.e., within ten months from now.)

Task 4d. Compare *B. fragilis* with loop-deleted OmpAs to the parental OmpA in a mouse abscess model.

Task 4e: Compare parental and *ompA* deletant strains in an invasion assay of brain microvascular endothelial cells.

Current status: Since there was no difference between the parental strain and the *ompA* deletant in the mouse abscess model, this task does not need to be completed. Similarly, since both the OmpA deletant (WAL 186) and the parental strain (WAL 108) were deficient in 1) adherence to intestinal epithelial cells and 2) biofilm formation compared to strain 638R (WAL 108 is a *thy*$^-$ deficient mutant of 638R, engineered to be more amenable to use in allelic exchange experiments) (Task 1D), there was no further need to test specific loop deletants.

Table 1: Strains and plasmids used in this study

	Description or relevant marker	**Source or reference**
Strains		
WAL 108	*B. fragilis* ADB77; TM400, ΔthyA, rifampicin^{R}	Malamy
WAL 186	*B. fragilis* ADB77 ΔompA	This study
WAL 361	*B. fragilis* ADB77 rec *ompA*	This study
	E.coli Top 10, host strain for cloning	
	E.coli Top 10/ pET27b(+)::ompA; kan ^{R}	Stratagene
	E.coli Top 10/ pET27b(+)::ompA FLAG; kan ^{R}	This study
	E.coli XL-1 Blue, host strain for cloning	Stratagene
	E.coli XL-1 Blue/pBR322; tet ^{R} amp ^{R}	This study
	E.coli XL-1 Blue/ pBR322::upompAdown; amp ^{R}	This study
	E.coli XL-10-Gold, Ultracompetent cell	Stratagene
	*E. coli/*pK2317	Malamy
	E. coli/TOPO::BamH1up*ompA*partialAATII	This study
	E. coli/TOPO::BamH1up*ompA*partialFLAGGAATII	This study
	E. coli/TOPO::BamH1up*ompA*partialHIS6AATII	This study
	E. coli/TOPO:: AATII-*ompA*partial*Sal1--HindIII	This study
	E. coli/pBR322:: BamH1up*ompA*partialHIS6AATII-*ompA*partial-HindIII	This study
Plasmids		
	pET27b(+); kan ^{R}, expression vector	Tomzynski
	pET27b(+)::*ompA*; kan ^{R}	This study
	pET27b(+)::*ompA* FLAG; kan ^{R}	
	pBR322; tet ^{R} amp ^{R} , cloning vector	
	pBR322::up*ompA*down; amp ^{R}	

APPENDICES:

Cytokine release and expression induced by OmpA proteins from the Gram-negative anaerobes, *Porphyromonas asaccharolytica* and *Bacteroides fragilis*

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Running head: Cytokines induced by OmpA from anaerobes

Keywords: *Porphyromonas asaccharolytica*; *Bacteroides fragilis*; OmpA; Cytokine

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Abstract

OmpA proteins from Gram-negative anaerobes *Porphyromonas asaccharolytica* and *Bacteroides fragilis* induced release and expression of IL-1α, TNF-α, IFN-γ, IL-6, and IL-10 from murine splenocytes *in vitro* in a dose-dependent fashion. The release of the cytokines induced by *B. fragilis* Bf-OmpA was at much lower levels compared to *P. asaccharolytica* Omp-PA; Bf-OmpA didn't induce release of IL-10. Omp-PA and Bf-OmpA were able to

up-regulate mRNA expression of the tested cytokines. Results obtained with refolded Bf-OmpA were similar to those with native Bf-OmpA. The data presented in this research demonstrate for the first time that Omps from anaerobic bacteria can induce the release of cytokines, suggesting that Omp-PA and Bf-OmpA may play important roles in the pathogenic processes of these bacteria.

1. Introduction

Porphyromonas asaccharolytica is a Gram-negative non-sporulating anaerobic rod which was formerly part of the genus *Bacteroides* [1]. Infections by this pathogen are associated with soft tissue infections below the waist, foot ulcers, appendiceal abscesses and empyema [2]. *P. asaccharolytica* was also implicated in cases of bacteremia [3] and in a left cardiac myxoma [4]. *Bacteroides fragilis*, a non-spore-forming, Gram-negative rod, is the most common anaerobic organism isolated from clinical infections. It is frequently associated with extraintestinal infections such as abscesses and soft-tissue infections, as well as diarrheal diseases in animals and humans. *B. fragilis* has the ability to invade the host immune response which contributes to the virulence of the bacterium. It's capsule can mediate resistance to complement-mediated killing and to phagocytic uptake [5].

The outer membrane of Gram-negative bacteria acts as a dynamic interface between the cell and its surroundings, and the importance of this interaction in both pathogenesis of the infection and immune response of the host has been investigated. The major components of the outer-membrane proteins in *P. asaccharolytica* and *B. fragilis* are proteins of the OmpA family, Omp-PA and Bf-OmpA, respectively. The importance of OmpA in the pathogenic process has been increasingly recognized. OmpA has been implicated in the invasion of brain microvascular endothelial cells (BMEC) [6,7] and has been shown to contribute to the ability of *E. coli* to cross the blood-brain barrier [8].

Outer membrane proteins of the porin class, in general, possess a variety of immunomodulatory and procoagulant activities [9-12]. Omp-PA of *P. asaccharolytica* is the major porin protein of that organism [13]. In contrast, the Bf-OmpA protein does possess some pore-forming activity in liposomes but is not the major porin protein of *B. fragilis* [14]. Non-toxic concentrations of porins from a variety of organisms stimulate the synthesis and release of platelet-activating factor and promote proinflammatory and immunomodulatory cytokine release from immunocompetent cells or other cellular sources [15]. Porins can induce the release of TNF-α, IL-1α, and IL-6 by human monocytes and of IFN-γ and IL-4 by human lymphocytes. This was seen with porins from *Salmonella*

typhimurium [11,16,17], *Pseudomonas aeruginosa* [10,15], and *Pasteurella multocida* [12]. The OmpA-like porin from *Acinetobacter* spp. stimulates the secretion of gastrin and IL-8 [18], and *Shigella dysenteriae* type 1 porin induces the release of nitric oxide and IL-1 [9]. The accumulated evidence clearly indicates that porins mediate release of cytokines and other proinflammatory factors, and that this activity may vary from one porin type to another.

The aim of this study was to investigate and compare the abilities of the OmpA proteins *P. asaccharolitica* and *B. fragilis* to induce the release of different cytokines by murine splenocytes in vitro. The significance of this study is due to the fact that Bacteroides and anaerobes in general are quite different from other aerobic microorganisms.

2. Materials and methods

2.1. Animals

BALB/cByJ male mice 8 weeks old were used. The animals were housed at constant temperature (20 ± 2°C) under a fixed 12 h light–dark cycle with free access to food and water.

2.2. Preparation of Omp-PA and Bf-OmpA

P. asaccharolytica ATCC 25260 and *B. fragilis* ATCC 25285 were grown anaerobically for 72 h and 96 h, respectively, at 37 °C in Brucella Broth medium supplemented with hemin and vitamin K (5 and 1 μg/mL). The cells were harvested by centrifugation (Sorval RC-2B) at 8000 rpm for 20 min and washed once in 10 mM Tris-HCl buffer, pH 7.4, containing 10 mM $MgSO_4$. Cells were broken by sonication at a power level of 7.5 for 16 min (GAMMA1-20 THRIST). The suspension was centrifuged at 3000 rpm for 5 min to remove whole cells and cell debris. The supernatants contained the cell envelope and cytoplasm. The inner membrane of *B. fragilis* was solubilized by adding 2% Triton X-100 containing 10 mM $MgCl_2$ and 10 mM HEPES to the supernatant, incubating for 30 min at room temperature, and then centrifuging at 15000 rpm. The inner membrane of *P. asaccharolytica* was solubilized by adding 0.5% N-laurylsarcosine to the

cell envelope preparation. The mixture was incubated for 30 min at room temperature and then centrifuged at 15,000 rpm for 1 h. The resulting pellet containing the crude outer membrane (OM) was washed once with 10 mM Tris-HCl, 10 mM $MgSO_4$, pH 7.4, and then lyophilized. Fifty mg of lyophilized OM was suspended in 20 mM Tris-HCl, pH 7.4, containing 0.3% LDS and 5 mM EDTA to remove loosely associated OM proteins. After incubation for 30 min at 4 °C, the suspension was centrifuged at 15,000 rpm for 1 h. Omp-PA from *P. asaccharolytica* and Bf-OmpA from *B. fragilis* were extracted by resuspending the pellet in 10 mM Tris-HCl, pH 7.4, containing 2% LDS and 5 mM EDTA, incubating for 30 min at 4 °C and then centrifuging at 15,000 rpm for 1 h. The supernatants contained Omp-PA from *P. asaccharolytica* and Bf-OmpA from *B. fragilis*. Lipopolysaccharide (LPS) contamination in the final preparations was detected by the Limulus test [19] and was 10 pg/10 μg porins. To eliminate any biological effect of LPS, proteins were incubated for 1 h with 5 μg/ml polymyxin B (Sigma) at room temperature [20]. In all of the tests performed, the porin with polymyxin B gave the same results as the porin alone (data not shown).

2.3. SDS polyacrylamide gel electrophoresis

The supernatant containing the OmpA protein was subjected to SDS-PAGE in a modified Laemmli gel with an acrylamide:bisacrylamide ratio of 30:0.8 and an acrylamide concentration of 12.5% w/v for the running gel and 5% w/v for the stacking gel. Molecular weights were determined using molecular weight standard proteins (#161-0304, BioRad).

2.4. Passive elution

Bands corresponding to Omp-PA (37 kDa) and Bf-OmpA (36.8 kDa) were cut out from the gel, placed in dialysis bags and mashed manually. Buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS, and 3 mM sodium azide was then added and the preparations were dialyzed against the same buffer overnight at 4 ºC. The gel/buffer mixtures were centrifuged to remove the gel traces, which were re-extracted with the same buffer and recentrifuged. In order to remove the detergent traces the supernatants from both extractions were combined and redialyzed

against buffer containing 20 mM Tris-HCL and 3 mM sodium azide for 4 days at room temperature, with frequent changes of the buffer, and then lyophilized.

2.5. Cloning and expression of B. fragilis ompA *in* E. coli *and purification of recombinant Bf-OmpA from inclusion bodies*

Primers to amplify the Bf-*ompA* gene from genomic DNA were constructed with appropriate restriction sites (NdeI and BamH1) for subsequent cloning of the PCR product into pET-27b(+). The primers used are indicated in Table 1. The cycles were 95 ºC – 3 min, followed by 30 cycles of 95 ºC – 45 sec, 62 ºC – 30 sec, 72ºC – 4 min, and finally 72 ºC – 5 min. One microgram each of plasmid and PCR product were digested with BamHI and NdeI as directed by the manufacturer. Both the digested pET-27b(+) and the digested Bf-*ompA* were purified from a 1% agarose gel. The gel slices containing the desired bands were melted at 65 ºC for 5 min. Fifty nanograms of digested vector and 100 ng of digested insert were mixed with 400 U of T4 ligase in T4 ligase buffer (20 ul total volume) for 4.5 h. Five microliters of the ligation mixture was used to transform XLBlue MRF` and the reaction was plated on LB agar with kanamycin. Transformants were screened by PCR and verified by miniprep plasmid analysis.

Purified plasmid DNA was used to transform BL21 according to the manufacturer's instructions and plated on LB agar with kanamycin. Overnight culture (0.5 mL) was used to inoculate 1 L of LB with kanamycin and grown in a 37 ºC shaker until the OD600 reached 0.4 U. The culture was induced at this point by adding IPTG to a final concentration of 1 mM. Protein induction proceeded for 3 h, and then the cells were harvested by centrifugation for 20 min at 7000 rpm at 4 ºC. The cell pellet was washed with 20 mM Tris-HCl, pH 7.4, recentrifuged, and then frozen and stored at -20 ºC.

The frozen cell pellet from 1 L of induced culture was resuspended in 100 ml of 20 mM Tris-HCl, pH 7.4, with 100 µg/mL lysozyme. The suspension was sonicated at a power level of 4 for 9 min until homogenized. The lysate was centrifuged for 5000 rpm for 5 min at 4 ºC to pellet the inclusion bodies containing the recombinant Bf-OmpA. The inclusion bodies were washed twice with 100 ml of 20 mM Tris-HCl, pH 7.4, supplemented with 10 mM EDTA,1%Triton X-100. The washed inclusion bodies were frozen at -20 ºC.

2.6. Refolding of over-expressed Bf-OmpA

The frozen inclusion bodies were resuspended to 20 mg/mL (0.48 mM) in 8 M urea, 10 mM borate, 2 mM EDTA, pH 10. Twelve milligrams of the resuspended protein was incubated with 16 mM 3-14 zwittergent for 4 days at 37ºC. A band indicating the refolded Bf-OmpA was cut out of the gel, passively eluted, and then diluted in the cell culture at the appropriate concentration (0.1±10 mg ml^{-1}) for the cytokine assays.

2.7. Murine splenocyte preparation

Murine splenocytes were prepared according to conventional procedures from aseptically removed mouse spleens (known to contain more than 97% lymphocytes of different types). Erythrocytes were lysed using 0.155 M NH_4Cl, washed three times in RPMI 1640 medium (Labtek Laboratories, Eurobio, Paris, France), resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), glutamine (2 mM), penicillin (100 U ml^{-1}), and streptomycin (100 U ml^{-1}) at a concentration of 3 x 10^6 cells ml^{-1}, and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.8. Murine splenocyte stimulation

Murine splenocytes resuspended at 3 x 10^6 cells ml^{-1} in complete medium were divided into aliquots to be treated or left untreated. The proteins were prepared in pyrogen-free distilled water and then diluted in the cell culture at the appropriate concentration (0.1±10 μg ml^{-1}). The incubation time was 24 h and 48 h for cytokine assays and 3 h for mRNA analysis.

2.9. Cytokine assays

All assays were carried out with 3 x 10^6 cells ml-^{1} stimulated with various concentrations of Omp-PA, native Bf-OmpA, and recombinant Bf-OmpA, and were incubated at 37 °C in 5% CO_2 for 24 and 48 h. At specified time intervals, cell viability was checked by Trypan blue exclusion test. Culture supernatants were harvested by centrifugation and stored at
-20 °C until assayed for cytokines. All measurements were carried out using monoclonal

antibodies. IL-1α, IL-6, TNF-α, IFN-γ, and IL-10 were measured by immunoenzymatic methods (ELISA kits of Invitrogen, Biosource, Worcester, MA).

2.10. Lactate dehydrogenase (LDH) assay

LDH assay was carried out according to the manufacturer's instructions using a Cytotoxicity Detection kit (Promega). LDH is a stable cytoplasmic enzyme present in all cells and is rapidly released into cell culture supernatant upon damage of the plasma membrane. LDH activity was determined by a coupled enzymatic reaction whereby the tetrazolium salt (INT) was reduced to formazan. An increase in the number of dead or damaged cells results in an increase in LDH activity in the culture supernatant.

2.11. RNA isolation and cDNA preparation

The concentrations of Omp-PA used in the assay were 0.1 μg/ml for TNF-α, 1 μg/ml for IFN-γ and IL-10, and 5 μg/ml for IL-1α and IL-6. The concentrations of native and recombinant Bf-OmpAs used in the assay were 0.1 μg/ml for IL-1α and IFN-γ, 1 μg/ml for TNF-α, and 5 μg/ml for IL-6. Non-stimulated cells were used as negative controls. The stimulated and the non-stimulated cells were collected after 3 h of incubation. Mouse β-actin was used as an internal standard. Total RNA was extracted according to the method of Chomczynski and Sacchi [21]. The RNA pellet was resuspended in 75% ethanol, sedimented, vacuum-dried and dissolved in 50 μl of RNase free water. One μl of oligo (dT) (Promega Biotechnology, Madison, WI) was added to the suspension containing 2 μg of RNA and the mixture was heated at 70 °C for 5 min. After cooling on ice, the mixture was incubated for

2 h at 42°C with 14 μl of the following mixture: 20 mM dithiothreitol (Sigma, St. Louis, MO); 1 mM (each) dATP, dGTP, dCTP, and dTTP; 35 U of RNasin (Promega); and 525 U of Moloney murine leukemia virus reverse transcriptase (Promega) in reverse transcription buffer.

2.12. PCR procedure

The primer pair sequences were designed on the basis of published cytokine gene sequences as reported in Table 2. The primer sequences were complementary to sequences in the exons or spanned exon ± exon junctions and thus were RNA-specific. One µl of cDNA prepared as described above was amplified in the presence of 1 µl of 5' and 3' primers, 0.5 µl of dNTP (Promega), 2.5 µl of *Taq* DNA polymerase 10 x buffer (Promega) and 0.5 µl of *Taq* DNA polymerase (Promega) in a final volume of 25 µl. The PCR reactions were performed in a DNA thermal cycler (Perkin-Elmer-Cetus Instruments, Norwalk, CT). All PCRs started with a 3 min denaturation step that was followed by 35 cycles of 1 min of denaturation at
94 ºC, 1 min of annealing temperature, and 1 min of extension at 72ºC. A final 10 min at 72 ºC was used in all cases. Annealing temperature used for primers was as follows: IL-1α 60 ºC, TNF-α 60 ºC, IFN-γ 60 ºC, IL-6 60 ºC, IL-10 60 ºC, and β-actin 60 ºC. Twenty-five µl of the reactions was subjected on 1.5% agarose gel and the electrophoresis was performed at 100 V. One µg of GeneRuler, DNA Ladder Mix (#SM0331, Fermentas) was run in parallel as a molecular weight marker (providing bands at 100-1000 bp).

2.13. Statistics

The immunoenzymatic assays were carried out in triplicate and the results were expressed as the mean ± standard deviation. Comparisons between tests were made by Student's t-test with statistical significance considered to be $p<0.05$.

3. Results

3.1. Purity of Omp-PA and Bf-OmpA preparations

SDS-PAGE analysis of the purified proteins is shown in Fig. 1. SDS-PAGE of Omp-PA showed one band with a molecular weight of 37 kDa (Lane 3, Fig. 1), as reported previously [13]. SDS-PAGE analysis of Bf-OmpA demonstrated one band of 36.8 kDa (Lane 4, Fig. 1), as previously reported [14]. The Limulus test indicated that LPS

3.2. Cloning and expression of B. fragilis ompA *in* E. coli *and purification of OmpA from inclusion bodies*

IPTG induced *E. coli* BL21 harboring plasmid pET-27b(+)-*ompA* produced inclusion bodies that contained mostly Bf-OmpA (Fig. 2).

3.3. Refolding of B. fragilis *OmpA from inclusion bodies*

Densitometric analysis refolded Bf-OmpA indicated that maximal results were achieved with 16 mM zwittergent 3-14 after incubation for 5 days at 37 °C. Approximately 51% of the overexpressed protein could be refolded under these conditions. The refolded Bf-OmpA migrated at the same apparent molecular weight of 36.8 kDa as the native Bf-OmpA (Fig. 2). It was shown earlier that the refolded Bf-OmpA has similar activity to the native protein in the liposome assay [14].

3.4. Release of IL-1α, TNF-α, IFN-γ, IL-6, and IL-10 from murine splenocytes induced by Omp-PA from P. asaccharolytica and native and recombinant Bf-OmpA from B. fragilis

The highest release of the cytokines from murine splenocytes induced by Omp-PA and both native and recombinant Bf-OmpAs was observed after 48 h of incubation. Cells stimulated with 5 μg/ml Con A were used as positive controls (data not shown), and the non-stimulated cells served as negative controls. LDH levels presented in the supernatants of stimulated cells were similar to those detected in the supernatants of non-stimulated cells, suggesting that cytokine release was not due to cell lysis (data not shown). Omp-PA induced high level secretion of the pro-inflammatory cytokines IL-1α, TNF-α, IFN-γ, IL-6, and anti-inflammatory cytokine IL-10 in a dose-dependent fashion and at a concentration ranging from 0.1 μg/ml to 10 μg/ml. The amounts (pg/ml) of each released cytokine are demonstrated in Fig. 3. The highest levels of the cytokine secretion were observed with the Omp-PA concentrations of 0.1 μg/ml for TNF-α, 1 μg/ml for IFN-γ and IL-10, and 5 μg/ml for IL-1α and IL-6. Concentrations higher than those caused a decrease in cytokine production, and a concentration lower than 0.1 μg/ml showed no significant effect. Both native and refolded recombinant Bf-OmpAs were able to regulate release of IL-1α, TNF-α,

IFN-γ, and IL-6 but in much lower levels compared to those obtained using Omp-PA. Both native and recombinant Bf-OmpAs had no significant effect on the release of IL-10 under the same experimental conditions. The highest levels of the cytokine secretion were observed with the protein concentrations of 0.1 μg/ml for IL-1α and IFN-γ, 1 μg/ml for TNF-α, and 5 μg/ml for IL-6.

3.5. Cytokine mRNA expression induced by Omp-PA from P. asaccharolytica and native and recombinant Bf-OmpA from B. fragilis

Expression levels of IL-1α, TNF-α, IFN-γ, IL-6, and IL-10 mRNAs were evaluated by treating murine splenocytes with Omp-PA and native and recombinant Bf-OmpAs in concentrations that showed maximum release of each cytokine as measured by ELISA kits (Fig. 3). The mRNA levels of all five cytokines, IL-1α, TNF-α, IFN-γ, IL-6, and IL-10, were increased upon stimulation of the cells by adding appropriate concentrations of Omp-PA (Fig. 4). The mRNA levels of the cytokines IL-1α, TNF-α, IFN-γ, and IL-6, expressed by stimulation of the cells with native and recombinant Bf-OmpAs, were similarly increased, but were found to be lower compared to those expressed by Omp-PA. Very low expression of
IL-10 mRNA was detected under the same experimental conditions. The non-stimulated cells did not show increased mRNA expression of the tested cytokines (Fig. 4).

4. Discussion

In this study, we investigated the ability of the OmpA proteins from *P. asaccharolytica* and *B. fragilis* to trigger release and expression of proinflammatory and immunoregulatory cytokines IL-1α, TNF-α, IFN-γ, IL-6, and IL-10 by murine splenocytes in vitro. Both native and refolded recombinant *B. fragilis* Bf-OmpA which are proved to be functionally identical [14] could elicit the cytokine release, but we were not able to refold the Omp-PA, and therefore, could not test it in this assay. The main reason for using recombinant refolded Bf-OmpA was to avoid the possibility that any capsule contamination in the purified protein preparation may be responsible for the cytokine

production [5]. The cytokine release proceeded in a dose-dependent fashion with different concentrations of porins needed for the maximum release of each cytokine. The release of IL-1α and IL-6 stimulated by Omp-PA was relatively low compared to the high-level production of TNF-α, IFN-γ, and IL-10. The results obtained cannot be attributed to the contaminating LPS (10 pg/10 μg of porin) in the porin preparations, because this trace amount of LPS had no ability to induce any cytokine production (data not shown). Moreover, porins were incubated with polymyxin B to neutralize biological activity of lipid A [16]. It is proved that the porin-polymyxin complex has the same activity in the induction of the cytokine secretion as the porin preparations alone, while the LPS-polymyxin complex is inactive [17].

Interestingly, Omp-PA was able to induce cytokine release at much higher levels compared to those obtained with native and recombinant Bf-OmpAs. We were initially surprised by this because *P. asaccharolytica* is considered to be less pathogenic than *B. fragilis*. However, mice were used as the experimental animals, and *B. fragilis* is part of mouse intestinal microflora. Thus the mouse was potentially immunized to the Bf-OmpA [22], which may explain why the stimulation of murine splenocytes by *B. fragilis* porin did not result in high-level secretion of cytokines.

Alternatively, perhaps other porin proteins present on the *B. fragilis* outer membrane may have more cytokine-stimulating activity. For example, the *B. fragilis* Omp-200 has more pore-forming activity than OmpA [23]. On the other hand, only one active monomeric porin (Omp-PA) was isolated from the outer membrane of *P. asaccharolytica*.

As expected, exposure to Omp-PA and native and recombinant Bf-Omp resulted in increased levels of IL-1α, TNF-α, IFN-γ, IL-6, and IL-10 mRNA expression. Results obtained in RT-PCR analysis corresponded to those measured by ELISA kits. For comparison purposes, we also checked the ability of another outer-membrane protein, Omp-EA, to induce the release and expression of IL-6 and IFN-γ. Omp-EA is an outer membrane protein from *Erwinia amylovora*, a pathogen which infects only plants [24]. As

expected, Omp-EA acted as a negative control and did not induce release of the tested cytokines (data not shown).

Recent reports suggest that the ability to induce cytokine production by the porins is dependent on the existence of the externally exposed loops that have been described extensively [25]. We have recently described these loops in monomeric porins from *Acinetobacter baumannii* [26], *B. fragilis* [14], and *P. asaccharolytica* [13]. Future work will include studying the role of the external loops of *P. asaccharolytica* Omp-PA and *B. fragilis* Bf-OmpA in induction of cytokine release.

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Table 1.

Primers used for the pET cloning

	Primer sequence (5` to 3`)
OmpAfwd	TGTTCATATGCAGCAGACTACAATTACGGGAT
OmpArev	AAGTGGATCCTTATTTAACAGACTCTACTAATA

Table 2

Primer sequences used for RT-PCR

Cytokine	Oligonocleotide sequence
IL-1α	5`AAGATGTCCAACTTCACCTTCAAGGAGAGCCG3` 5`AGGTCGGTCTCACTACCTGTGATGAGTTTTGG3`
TNF-α	5`TTCTGTCTACTGAACTTCGGGGTGATCGGTCC3` 5`GTATGAGATAGCAAATCGGCTGACGGTGTGGG3`
IFN-γ	5`TGCATCTTGGCTTTGCAGCTCTTCCTCATGGC3` 5`TGGACCTGTGGGTTGTTGACCTCAAACTTGGC3`
IL-6	5`ATGAAGTTCCTCTCTGCAAGAGACT3` 5`CACTAGGTTTGCCGAGTAGATCTC3`
IL-10	5`CTGGAAGACCAAGGTGTCTAC3` 5`GAGCTGCTGCAGGAATGATGA3`
β-actin	5`GTGGGCCGCTCTAGGCACCAA3` 5`CTCTTTGATGTCACGCACGATTTC3`

Figure legends

Fig. 1. Electrophoretic pattern of the purified porins from *P. asaccharolytica* ATCC 25260 and *B. fragilis* ATCC 25285. Lane 1: molecular weight standards. Lane 2: purified sample of *P. asaccharolytica* porin (37 kDa). Lane 3: purified sample of *B. fragilis* porin (36.8 kDa).

Fig. 2. SDS-PAGE of recombinant Bf-OmpA refolded in zwittergent 3-14. Lane 1: standard proteins; Lane 2: recombinant Bf-OmpA recovered from inclusion bodies; Lane 3: refolded recombinant Bf-OmpA incubated with 16 mM (3-14) for 5 days at 37ºC.

Fig. 3. Release of IL-1α, TNF-α, IFN-γ, IL-6, and IL-10 from murine splenocytes stimulated by *P. asaccharolytica* Omp-PA, *B. fragilis* native Bf-OmpA, and *B. fragilis* recombinant

Bf-OmpA at different concentrations after 48 h of incubation. Each point represents the mean of three experiments ± standard deviations. Points designated by asterisks indicate statistically significant differences ($p<0.05$) versus non-stimulated cells.

Fig. 4. Cytokine mRNA expression induced by *P. asaccharolytica* Omp-PA and *B. fragilis* native and recombinant Bf-OmpAs in murine splenocytes. Control indicates the non-stimulated cells.

Fig. 1.

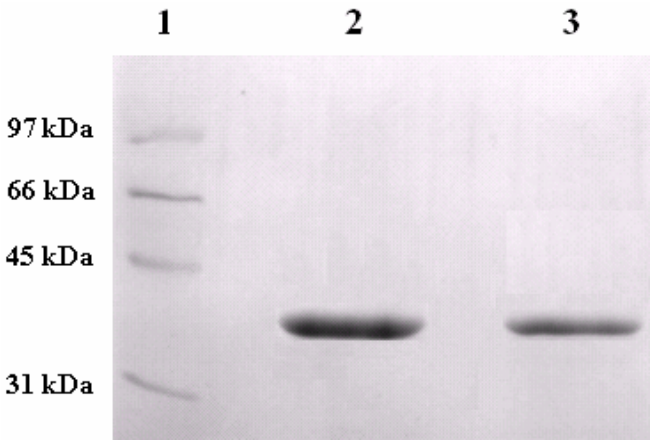


Fig. 2.

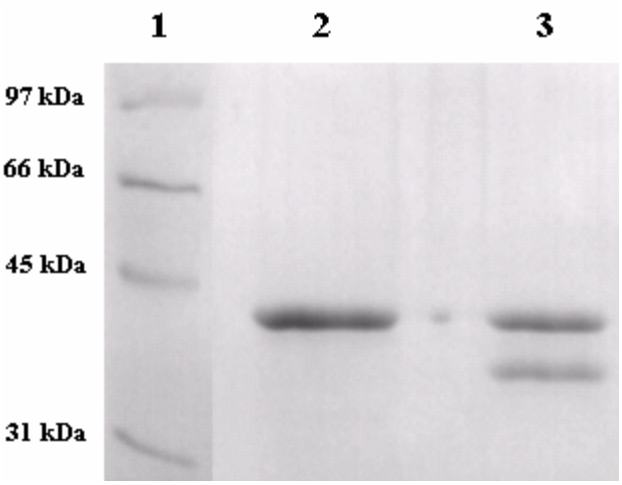


Fig. 3.

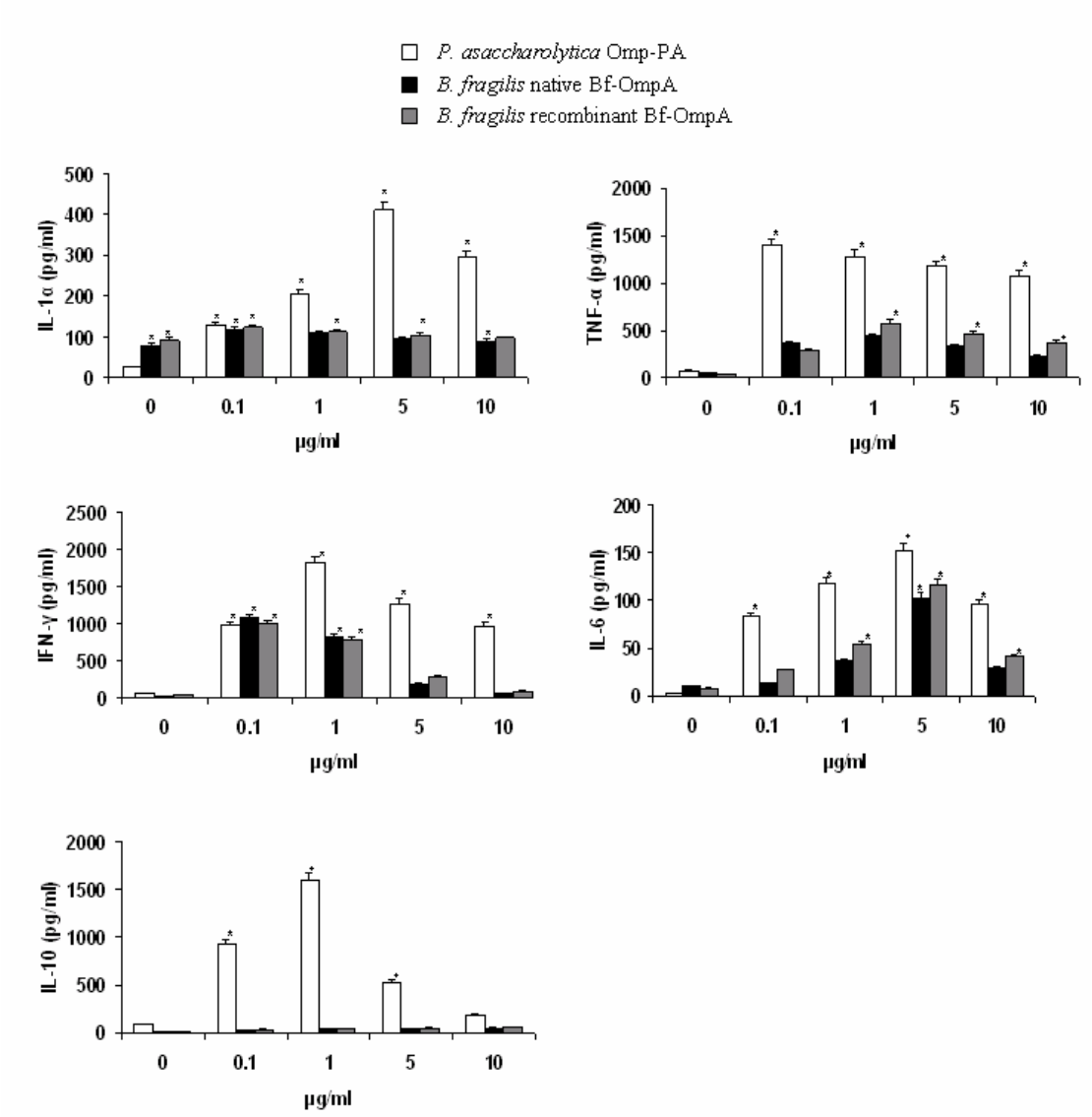
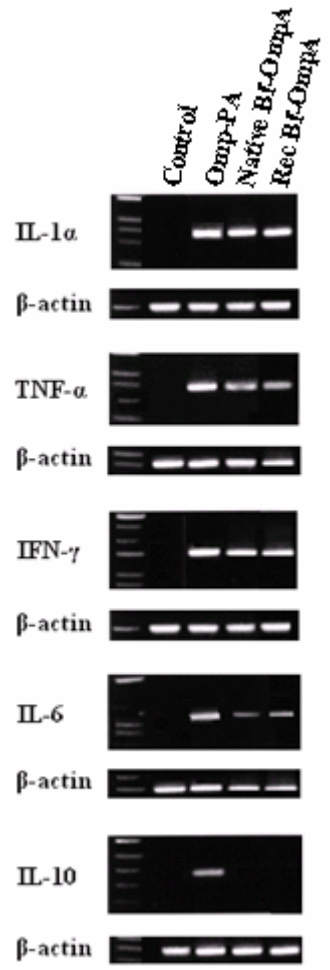

P. asaccharolytica Omp-PA
B. fragilis native Bf-OmpA
B. fragilis recombinant Bf-OmpA
IL-1α (pg/ml)
TNF-α (pg/ml)
IFN-γ (pg/ml)
IL-6 (pg/ml)
IL-10 (pg/ml)
0
0.1
1
5
10
μg/ml

Fig. 4.


Control
Omp-PA
Native Bf-OmpA
Rec Bf-OmpA
IL-1α
β-actin
TNF-α
β-actin
IFN-γ
β-actin
IL-6
β-actin
IL-10
β-actin

SUPPORTING DATA: All figures and tables are embedded in the text.

KEY RESEARCH ACCOMPLISHMENTS:

- Recombinant ompA reinserted into *B. fragilis* ompA deletant; proper sequence confirmed and OmpA protein expression verified
- pADB242a suicide vector modified and AatII site removed
- AatII site, AatII-FLAGG site, and Aat-His6 sites, respectively, inserted into Loop 2 of recombinant OmpA, cloned into TOPO and transformed into *E. coli* DH5alpha.
- SalII site inserted into Loop 3 of recombinant OmpA, cloned into TOPO and transformed into *E. coli* DH5 alpha.
- Cytokine assays were performed using recombinant OmpA

REPORTABLE OUTCOMES:

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